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14. ABSTRACT

In mice in which human androgen receptor (AR) replaces the endogenous murine gene, variation in the length of a polymorphic N-terminal polyglutamine tract affects initiation, progression and therapy response prostate tumors. This provides a genetic paradigm in which to dissect AR functions that determine response to therapy. We are studying the role of the AR Q tract in ligand-independent AR activation in vitro and in a mouse model with prostate cancer ontogeny similar to human. In the mouse model, molecular correlates of differential response to castration will be determined using bioinformatic analysis of microdissected tumor samples. In the first year of this award, we have constructed the necessary mouse strains to generate experimental animals, using a modified approach for optimal tumorigenesis. In the in vitro studies, we have shown that AR Q tract length confers differential activation in a promoter- and cell type-dependent manner. In addition, ARs with different Q tract lengths are differentially responsive to signal transduction cascades likely to be influential under castrate conditions. Furthermore, phosphorylation of AR by growth factor activation correlates inversely with Q tract length, providing a potential mechanism by which this polymorphism impacts AR function in castration recurrent prostate cancer.

15. SUBJECT TERMs
androgen receptor, polyglutamine tract, mouse models, response to therapy

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INTRODUCTION

Androgen, acting via the androgen receptor (AR), is central to prostate biology, and genetic variation in AR may impact development and/or progression of prostate cancer (PCa). Because tumors rely on androgen, therapy targets this signaling pathway and although initially successful, tumors ultimately become resistant to treatment. In these castration-recurrent tumors, AR levels remain high and AR signaling persists, implying that disease is independent of androgen but dependent on AR (1). To examine the role of AR in prostate cancer, we created a mouse model by germ-line gene targeting in which human AR sequences replace those of the mouse (2). Since shorter length of an N-terminal glutamine (Q) tract has been linked to PCa risk, we created alleles with 12, 21 or 48 Qs to test this association. These three "humanized" (h/mAR) strains are physiologically normal. However, distinct allele-dependent differences in initiation and progression of cancer are evident upon activation of a prostate-targeted oncogene, and these differences also impact progression following androgen ablation, with each allele conferring a distinct response (3). Mice with the transcriptionally more active 12Q-h/mAR respond well to castration, whereas those with the weaker 48Q-h/mAR show no benefit from treatment. These mice provide a genetic paradigm in which to dissect functions of AR that determine response to therapy. Identifying distinct pathways following treatment may reveal novel markers of response to therapy and suggest distinct strategies for optimal treatment. In this grant, we proposed to study the role of the Q tract in ligand-independent AR activation *in vitro* (Aim I) and, in a mouse model more similar to human cancer ontogeny, determine molecular correlates of differential response to castration (Aim II). In Aim III, results from mouse will be compared *in silico* to human datasets to identify pathways signifying differential response to therapy.

BODY

The grant outlined 4 tasks in the Statement of Work. We have completed the first, using a modified approach, have made substantial progress on the second, and have initiated the third. Relevant details are described below.

Task 1. Establish mouse lines with both conditional PTEN and Q tract variant humanized AR (h/mAR) alleles.

We initially proposed conditional PTEN deletion (4) to initiate prostate tumorigenesis in a C57BL/6 mouse background, for each of the three h/mAR alleles (12Q, 21Q or 48Q). Task 1 establishes the necessary compound lines to breed experimental animals. As we were starting, results from other investigators began to suggest that: a) the C57BL/6 background is resistant to PCa and FVB is preferable (5); b) conditional PTEN deletion is not uniform in the prostate, leading to potential difficulties interpreting data (6). To circumvent these possibilities, we put the Q tract alleles on the FVB background, necessitating 5 backcross generations, and modified the oncogenesis strategy to initiate cancer by global inactivation of one PTEN allele and cooperation of a prostate-targeted ETV1 fusion gene (7). This approach will provide more uniform oncogenesis than cre-mediated prostate-specific deletion of PTEN proposed before. Creation of the breeder strains differs, but overall mouse numbers and experimental design are

similar subsequently. One constraint is that the deleted PTEN allele must be introduced via the sire since female PTEN heterozygotes are nearly infertile. Below is the modified procedure; animal approval has been obtained from the U of M's UCUCA and from the DOD MRMC Animal Use Committee.

First we backcrossed the h/mAR mice onto the FVB background. All other mice needed (PTEN-floxed, EIIA-cre, and ARR2PBi-ETV1 tg) already are available on the FVB background. Next we created three strains doubly homozygous for one of the h/mAR alleles and carrying the ARR2PBi-ETV1 prostate-specific transgene **(A)**. Second, we are crossing these mice to PTEN heterozygotes, created by crossing PTEN-floxed mice with mice transgenic for EIIA-cre **(B)**. Mouse crosses necessary for breeding and generating experimentals **(C)** are presented below.

For each Q tract allele, 3 experimental groups (**n=8**) will be compared – **pretreatment** (12 weeks of age), **untreated** (intact at 24 weeks) and **treated** (castrated at 12 weeks, aged to 24 weeks). An equivalent number of littermate controls with one or both active PTEN alleles will be maintained as well. At the end point (Task 3), prostates will be microdissected, and similarly staged normal, premalignant and malignant cells will be pooled according to stage across mice of the same genotype and treatment group, for analysis of gene expression profiles.

A) Creation of mice homozygous for h/mAR^{nQ} and ARR2PBiETV1 tg:

F0 –breed ARR2PBiETV1 tg male X h/mAR$^{nQ/nQ}$ females
F1 – intercross tg positive males and females
F2 – select mice homozygous for AR allele (by genotype) and transgene (by progeny testing)

B) Creation of PTEN heterozygous mice (PTEN $^{-/+}$):

F0 - Cross PTEN$^{lox/lox}$ with female carrying EIIA-cre transgene (expresses in oocyte)
F1 – Cross male PTEN$^{-/+}$ with female FVB to insure deletion is not chimeric
F2 – Maintain PTEN$^{-/+}$ line by continuing to cross males back to FVB females

C) Generation of experimental mice:

Cross male PTEN$^{-/+}$ with female h/mAR; ETV1 tg.
If the female is homozygous for both AR and ETV1, half the males will be experimentally useful.
If the female is heterozygous for the transgene, ¼ of the males are useful.

For each Q tract allele, we will need to obtain 30 experimental and 30 control mice. We currently have some experimental animals for each AR allele, and expect to generate all within the 20 months originally projected

Task 2. Determine the role of the Q tract in ligand-independent androgen receptor (AR) activation in vitro.

The Q tract influence we noted in mouse PCa (3) suggests differences in AR strength may represent an important aspect of refractory disease. Short Q tract ARs have been noted to be transcriptionally more active than wild type at low androgen levels due to both greater intramolecular AR interaction between the N and C terminus (N/C interaction) and to greater

coactivator recruitment (8). AR can also be activated in the absence of androgen by growth factors, particularly IGF, EGF, cytokines (IL-6), PKA and tyrosine kinase receptors such as Her2/neu (9, 10). Whether growth factor signaling pathways are also sensitive to Q tract length has not been previously examined. To further our understanding of the role of the Q tract in castration-resistant prostate cancer, Task 2 is to examine: a) cell-type and promoter-specific differential activation by transfected 12Q, 21Q and 48Q AR cDNAs in the presence of low or no hormone, and b) ligand-independent differential activation elicited by co-expression of a constitutively active growth factor, in low or no hormone. To test central signal transduction pathways rather than specific factors, we proposed to use constitutively active MEKK1, but switched to constitutively active Raf for even broader activation (11). Our overall hypothesis is that shorter Q tract ARs are hypersensitive to low ligand and show greater activation by growth factors, perhaps by differential post-translational modification. Furthermore, this greater AR activity under castrate conditions promotes more differentiated and slower tumor growth.

To test whether differential AR activation dependent on Q tract length varied with cell type and promoter, we tested transactivation of 12Q, 21Q and 48Q AR cDNAs in 2 different cell lines and for 3 different promoters. The cell lines used were RWPE-1 cells, which are immortalized normal prostate (12), and PC-3, the classic late-stage PCa cells. Both cell lines are human in origin and do not express endogenous AR. For promoters, we tested the hallmark AR-responsive prostate-specific antigen (PSA) enhancer/promoter, which is a complex promoter with multiple binding sites for AR as well as other transcription factors (13). We also tested two distinct androgen responsive elements – the consensus inverted repeat that is also recognized by other steroid receptors (HRE3) and a related direct repeat that is more AR-specific in binding (HRE2) (14). The PSA promoter and multimerized HREs (3XHRE3, 4XHRE2) were tested before a minimal *tk* promoter driving a luciferase reporter gene in transient tranfection.

To test ligand sensitivity, we used the non-metabolizable synthetic androgen, R1881, at 0.01 and 1 nM concentrations, representing castrate and intact levels of androgen, respectively. Fig. 1 shows the variation in AR activation dependent on Q tract length under these conditions. The PSA reporter showed a modest transactivation difference for 12Q versus 48Q, but there was no significant difference in RWPE cells. This may in part be due to high basal activities and compensation for more minor AR differences conferred by other transcription factors binding the complex promoter. When the AR binding sites were examined independently, significant differences dependent on Q tract length were seen in PC-3 cells, at both low and high ligand concentrations, but these were not demonstrable in RWPE cells. This may indicate that in the cancer cell background, alterations in additional factors or coactivators confer greater sensitivity to the subtle difference in Q tract length. For the AR-selective HRE2 element, there is a significant difference between 12Q and 48Q AR activation that is more noticeable at the higher hormone concentration. Thus in this analysis, differences in AR activation that varied with Q tract length were significant in the cancer, but not in the normal, cell line, and were masked in the complex PSA promoter, likely due to compensatory effects of additional factors. We will in future test whether addition of specific coactivators (SRC-1, SRC-2 and SRC-3) can enhance the modest differences demonstrated in this experiment, perhaps especially at low ligand.

To test the extent to which ligand-independent growth factor activation was influenced by Q tract length, we tested activation in the presence of a constitutively active Raf-1 kinase to activate the

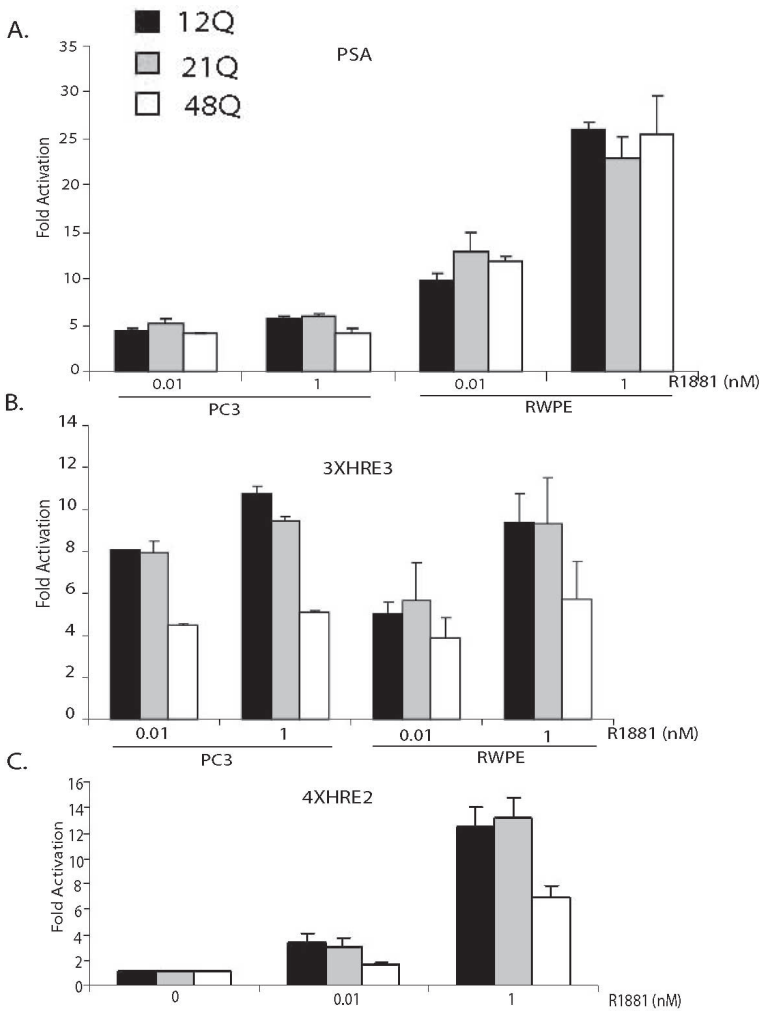

Fig. 1: Differential Q-tract Length Effects Dependent on Promoter- and Cell-context. Ligand sensitivity of AR Q-tract variants on PSA (A), 3XHRE3 (B) or 4XHRE2 (C) promoters was determined using PC3 (A, B and C) and RWPE (A and B) cells. Cells were transfected with 4 ng of receptor (12Q, 21Q or 48Q), 400 ng of reporter and 100 ng of promoterless renilla luciferase as an internal control. 24 hrs post transfection cells were fed with phenol red free media and treated with either methanol vehicle or R1881 (0.01 or 1 nM) for an additional 24 hrs. The histograms represent average values normalized to renilla from either three (A and B) or two (C) independent trials. Fold activation was determined by setting vehicle treated samples to 1.

MAPK cascade (11). Constitutively active (ca) Raf-1 kinase is a serine-threonine kinase that contains only the catalytic subunit and lacks the regulatory subunits. In the absence of ligand, negligible activity was obtained with (ca)Raf-1 for AR regardless of Q tract length (data not shown). However, in the presence of castrate levels of ligand (0.01 nM), (ca) Raf-1 kinase promoted greater transactivation with AR than ligand alone, and there was a trend for Raf activation to be inversely correlated with the AR Q tract length (Fig. 2). On the PSA reporter (Fig. 2A), addition of (ca) Raf-1 kinase enhanced 12Q-AR activation two-fold over that obtained with 0.01 nM R1881; the empty vector control showed little effect. 12Q-AR showed the greatest additional activation by Raf in both PC-3 and RWPE-1 cells, with more modest effects of (ca) Raf on 21Q-AR and 48Q-AR. For reasons that are unclear, there is greater experimental variability with added (ca) Raf, particularly on the PSA reporter, perhaps due to additional effects on other transcription factors binding this promoter. This is supported by the fact that on

the simple response element HRE3, there is reduced effect of (ca) Raf kinase, although it is statistically significant. Although there is little effect of Q tract length on HRE3 activation by Raf in PC-3 cells, in RWPE-1 cells there is a trend to inverse correlation with Q tract length, with no effect detected for 48Q AR.

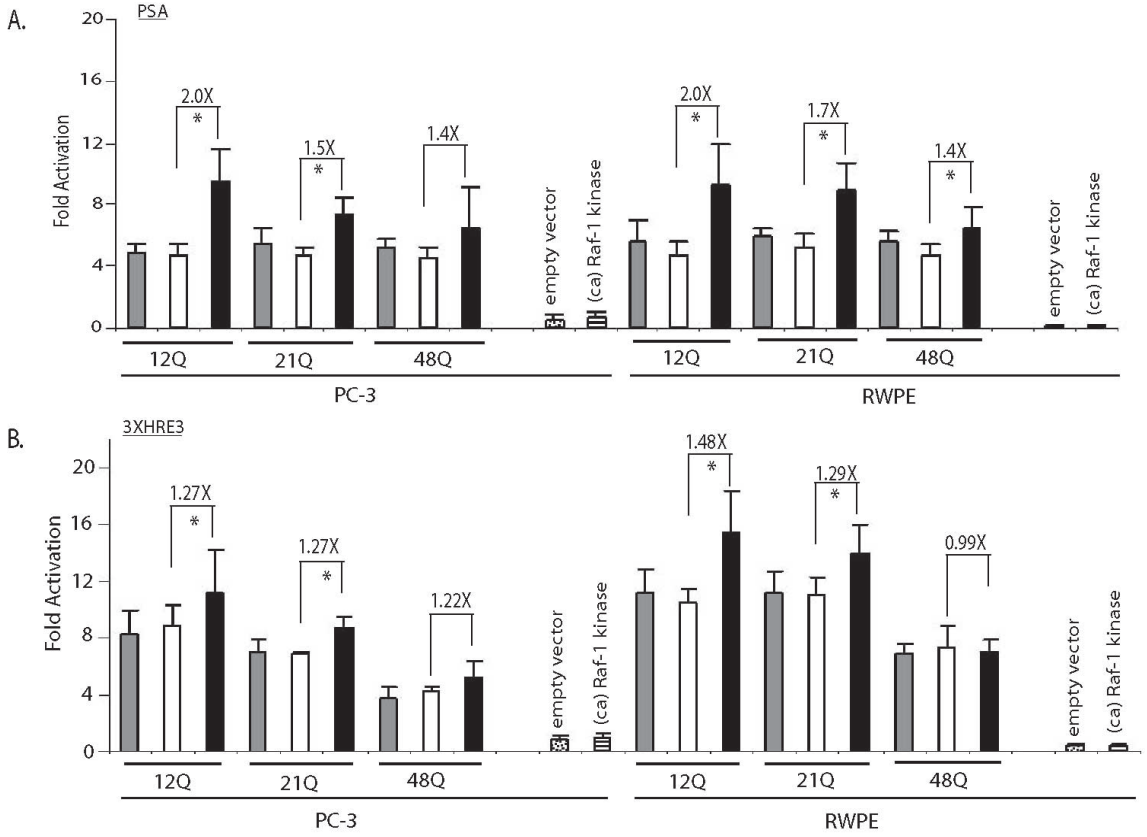

Fig. 2: Activation of AR Q tract variants by (ca) Raf-1 kinase and castrate levels of androgen. Shown is activation of the PSA (A) and 3XHRE3 (B) promoters. PC-3 or RWPE-1 cells were transfected with 25 ng receptor (12Q, 21Q or 48Q AR), 500 ng PSA (A) or 3XHRE3 (B) reporter, and with either 12.5 ng of (ca) Raf-1 kinase (black bars) or empty vector (white bars). Q-tract AR variants with hormone alone are represented by grey bars. 24 hrs post transfection cells were fed with phenol red free media and treated with either methanol vehicle or 0.01 nM R1881 for an additional 24 hrs. The graphs represent relative luciferase activities normalized to renilla from three independent trials. * p≤0.02, significant differences based on Anova single factor analysis. Horizontal striped bars and black dotted bars represent transfection of (ca) Raf-1 kinase or empty vector respectively, without AR.

Growth factor signaling often exerts post-translational protein modifications to influence target protein activity. Moreover, signaling through the MAPK pathway has been reported to induce androgen receptor phosphorylation (15). Therefore we examined the affect of activated Raf-1 kinase on the phosphorylation status of Q tract variant ARs. In particular, we examined the level of phosphorylation on serine residues at positions 81 and 650 in the AR. Ser81 is adjacent to the Q tract and thus its phosphorylation might be influenced by length of the tract, which is thought to serve largely as a flexible linker between functional AR domains (16). Ser650 is located in the hinge domain of AR, which is involved in DNA binding and contact with coregulators, and

has been reported to be phosphorylated in response to MAPK activation as well as activation by other non-androgen agonists such as forskolin (17, 18).

The Q tract variant ARs were transfected into PC-3 cells, along with (ca) Raf-1 kinase, and phosphorylation was assessed 24 hours later by Western blotting with specific AR phosphoserine antibodies. As shown in Fig. 3, phosphorylation of AR-Ser81 was moderately enhanced by addition of (ca) Raf-1 kinase, with a very slight trend to greater enhancement with shorter Q tract length. In contrast, phosphorylation of Ser650 showed more pronounced effects and distinctly greater phosphorylation for the shorter 12Q and 21Q ARs. This suggests that Q tract length may differentially impact phosphorylation within the hinge domain of AR, perhaps due at least in part to the greater N/C interaction promoted by the short Q tract. These results will be confirmed and the mechanistic basis elucidated in future experiments.

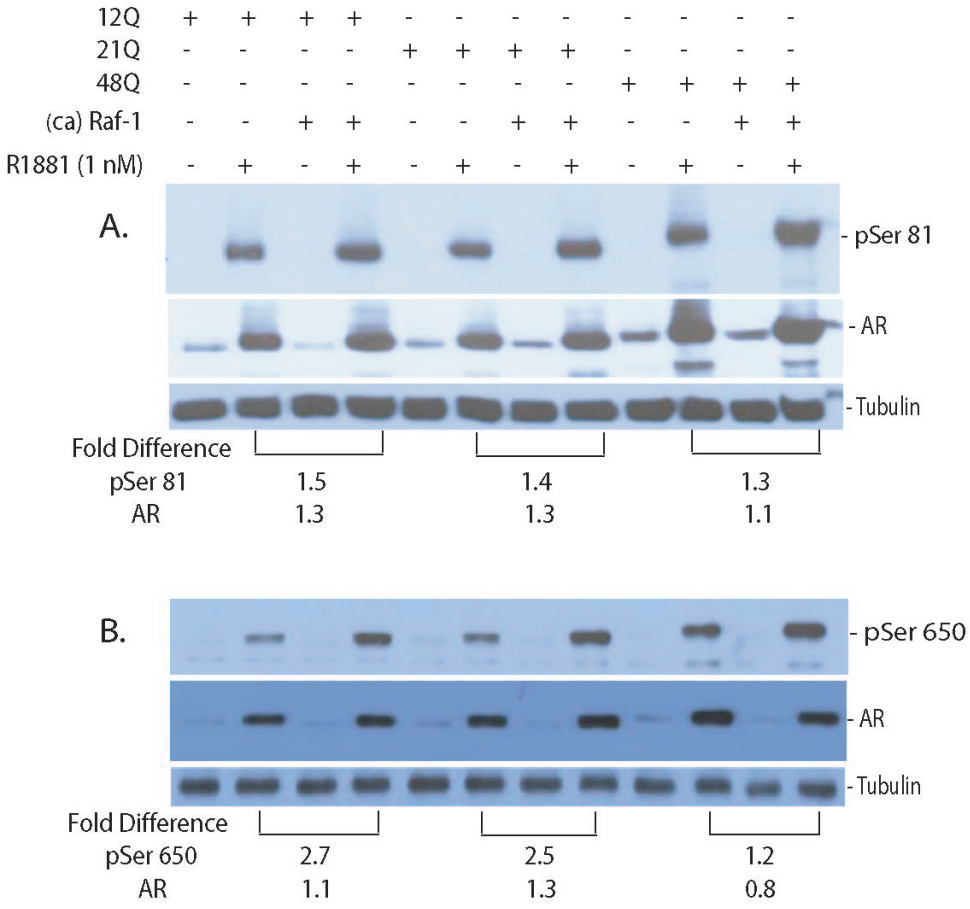

Fig. 3: Differential AR phosphorylation induced by (ca) Raf-1 kinase. Immunoblot analysis of whole cell lysates prepared from PC-3 cells, transiently transfected with 100 ng AR (12Q, 21Q or 48Q) and 100 ng (ca) Raf-1 kinase expression plasmids. 24 hrs post transfection cells were fed with phenol red free media and treated with either methanol vehicle or 1 nM R1881 for an additional 24 hrs. The top panel (A) was probed with anti-phospho AR (Ser 81) antibody, anti-AR (N20) and anti-tubulin as a loading control. The lower panel (B) was probed with anti-phospho AR (Ser 650) and anti-tubulin antibody. Image J software was used for quantitation of the blots.

Task 3. Determine the molecular correlates of androgen ablation response as affected by AR strength in prostate-specific PTEN-inactivated mice.

Part A of this task is to generate the experimental mice, and as mentioned above at the end of Task 1, we have begun to accrue experimental animals and expect to be on time with completion of this task.

KEY RESEARCH ACCOMPLISHMENTS

- The Q tract variant h/mAR mice have been back-crossed onto the FVB background to allow greater susceptibility to prostate cancer than on the C57BL/6 background.
- The compound ARR2PBi-ETV1 and Q-h/mAR strains, and PTEN heterozygotes, have been established on a FVB background; breeding of experimental progeny is in progress.
- 12Q, 21Q and 48Q AR cDNAs show differential activation in a promoter- and cell type-dependent manner.
- 12Q, 21Q and 48Q ARs are differentially responsive to signal transduction cascades likely to be influential under castrate conditions.
- Phosphorylation of AR by growth factor activation, particularly at Ser650, appears to correlate inversely with length of the Q tract.

REPORTABLE OUTCOMES

As yet no publications or abstracts have result from the first year of this award. I have discussed some of the preliminary results and approaches in seminars presented at the University of Minnesota, Department of Pharmacology (10/02/08) and at the University of Miami, Department of Molecular and Cellular Pharmacology (6/24/09). Some ongoing work was mentioned in an invited lecture for Women in Andrology at this year's Andrology Society Symposium, Philadelphia, 4/5/09.

CONCLUSION

In the first year of this DOD IDEA award, we have constructed the appropriate mouse strains to establish a unique mouse model in which to address the role of the AR Q tract in differential response to androgen ablation therapy. This model is optimized to circumvent previous problems due to strain background and lack of homogeneity in prostate cells, and will utilize a

genetic paradigm that is more similar to human prostate cancer ontogeny (heterozygosity for PTEN deletion and overexpression of ETV1) than the TRAMP model. Tumors from these mice will be assessed using bioinformatic means to determine pathways differentially utilized for good response compared to poor response to castration therapy.

As a complement to the in vivo studies, we have examined molecular mechanisms underlying differential activation conferred by Q tract variant ARs. We have demonstrated that ARs with different Q tract lengths are sensitive to promoter and cell type differences; this may impact diverging activities of AR during tumor progression dependent on Q tract length. Furthermore, we have shown that ARs with different Q tract lengths are differentially sensitive to growth factor signaling, demonstrated at both the level of transactivation in reporter assays and for phosphorylation of specific serines within AR. Subtle differences conferred by Q tract length may prove one of many factors that sum to significant affects in response to therapy.

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